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Doxorubicin-Induced DNA Degradation in Murine Thymocytes

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SUMMARY

Exposure of murine thymocytes to doxorubicin (Dox) (0.5–1.0 μM, 24 hr) triggered rapid DNA degradation, as indicated by the appearance of a major subdiploid population demonstrated by DNA flow cytometry. Electron microscopic comparison of samples with large subdiploid populations versus those with little or no such subset revealed significantly more cells with the characteristic features of apoptosis, the morphologically definable stage of programmed cell death. These features include unipolar condensed chromatin, zeiosis, and electron-dense cytoplasm. Dox-induced apoptosis occurred without prior S or G_2/M phase arrest or cell size increase. The subset most susceptible to Dox-induced apoptosis *in vitro* and *in vivo* was $CD3^-CD4^+CD8^+$. The same subset is affected by dexamethasone (Dex); as reported for Dex-induced apoptosis, actinomycin D and cycloheximide also blocked Dox-induced apoptosis. Thymocytes exposed to higher Dox concentrations (2–10 μM) did not have a subdiploid population. Although at 2–10 μM Dox significantly reduced cell numbers (probably as a result of necrosis), at least 5–10% of the population was viable at 24 hr. Thymocytes exposed to low concentrations of Dox (0.001–1.0 μM) plus Dex (0.1 μM) exhibited additive induction of apoptosis, whereas those exposed to high concentrations of Dox (2–10 μM) plus Dex were completely devoid of any evidence of apoptosis. These results indicate that the Dox-induced killing in thymocytes (mostly noncycling cells) occurs via different mechanisms depending upon the Dox concentration.

The two distinct mechanisms of chemotherapy-induced cell death, apoptosis and necrosis, exhibit significantly different morphological, biochemical, and biological characteristics. Steroids and topoisomerase inhibitors have been shown recently to kill tumor cells and noncycling thymocytes preferentially by the apoptotic mechanism (1–6). There is, however, conflicting evidence about the ability of Dox to induce apoptosis (3–6). The fact that Dox is therapeutically effective against an extremely wide spectrum of tumors is consistent with the possibility that it acts via multiple tumoricidal mechanisms. Thus, as a DNA-intercalating agent Dox causes unbalanced cell growth with G_2/M arrest and an associated increase in cell size, protein, and RNA content before cell death (7, 8). Cell killing by this Dox-mediated mechanism requires actively cycling cells; neither cells in stationary growth phase nor those whose growth cycle has been blocked by anguidine are killed (9, 10). In contrast, Dox-induced apoptosis of intestinal crypt cells is independent of the cell cycle (11).

Studies of apoptosis in thymocytes can provide additional information about the mechanism of Dox-induced cell death, because the majority of thymocytes are G_0/G_1 cells, which are predominantly in what has been termed a "half-activated" cell cycle state (12). Cells in this state presumably are primed to respond to the negative and positive pressures of the thymic microenvironment and either undergo PCD or differentiate into mature immunocytes. It is also known that thymocytes have a high content of topoisomerases, although a possible role for this enzyme in thymocyte PCD has not been suggested. It has been known for some time that apoptosis, the morphologically definable process of PCD, is a physiologically active mechanism in the thymus for deletion of autoreactive clones. The role of PCD in thymic clonal deletion has been extensively studied, and the potential of phenotypically defined thymocyte subsets to enter PCD is known. The exposure to self antigen in the context of major histocompatibility complex or another weak stimulus induces rapid apoptotic death of $CD4^+CD8^+$ cells in the thymus (13, 14), whereas exposure to the cytotoxic agent cisplatin failed to trigger PCD in rat thymocytes, although it initiated extensive apoptosis in a hepatoma cell line (15).

The present study demonstrates that Dox induces PCD in thymocytes over a narrow concentration range, whereas at higher concentrations it induces necrosis and blocks glucocorticoid-triggered apoptosis. The DP thymocytes ($CD4^+CD8^+$) were found to be the most susceptible population targeted by Dox *in vivo* and *in vitro*.

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ABBREVIATIONS: Dox, doxorubicin; Dex, dexamethasone; Act, actinomycin D; Clx, cyclohexamide; IL, interleukin; FCM, flow cytometry; PCD, programmed cell death; TCR, T cell receptor; DP, double-positive; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FITC, fluorescein isothiocyanate; TC, tri-colon.

Materials and Methods

Animals. Female C57BL/6NCr mice were obtained at 5 weeks of age from the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, and were used at 6–8 weeks of age.

Reagents. Dox (Adriamycin) was a gift from Adria Laboratories. Dex, Clx, and Act were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human IL-2 was a gift from DuPont de Nemours & Co (Glenolden, PA). Medium used in all experiments was RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine, 50 μg/ml gentamicin, 50 μM 2-mercaptoethanol, and 10 mM HEPES buffer (complete medium).

Cells and culture. Lymphocytes were prepared from thymus and spleen by gentle pressing of the organs against stainless steel screens. Single-cell suspensions were made after two washings and filterings through fine (200-mesh) stainless steel screens. Only samples with a viability of >95% (trypan blue exclusion test) were used. Suspensions were adjusted to 5×10^6 cells/ml in complete medium, and triplicate 200-μl aliquots were incubated in 96-well plates (Falcon, Lincoln Park, NJ) at 37° in 5% CO_2. Dox concentrations ranged from 10^{-3} μM to 10 μM. Dex was used at 0.1 μM, a concentration that is known to induce apoptosis (1). In some experiments 50 μg/ml Clx or 2 μg/ml Act was added to the cultures.

DNA FCM. After incubation, thymocytes were washed twice in PBS, fixed in cold 70% ethanol, and stained with propidium iodide (50 μg/ml; Sigma) in hypotonic citrate solution, with addition of 0.1% Nonidet P-40 (Sigma) and RNase (Sigma), for 1 hr before FCM analysis of cell cycle distribution. Ten thousand events were acquired on a FACScan (Becton Dickinson, San Jose, CA) in list mode with linear or logarithmic amplification, as suggested by Nicoletti *et al.* (16). Data were analyzed with Modfit (Verity, Topsham, ME) software for cell cycle distribution.

Agarose gel electrophoresis of DNA. Untreated and drug-treated thymocytes were collected by centrifugation and washed in PBS. Five million cells were resuspended in 500 μl of lysis buffer (10 mM Tris, pH 7.6, 25 mM EDTA, 400 mM NaCl, 1% sodium dodecyl sulfate, 200 μg/ml proteinase K). Cell lysates were incubated (50°, for 30 min) and centrifuged (13,000 × *g*, for 30 min). Supernatants were separated immediately from the viscous pellets containing large uncleaved chromatin. DNA fragments were precipitated from 450 μl of this supernatant with 1 M NaCl and 1.5 volumes of isopropanol at −70°, overnight. After centrifugation (13,000 × *g*, for 20 min), samples were washed with 70% ethanol, allowed to dry, resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA buffer with 5 units of RNase (total, 25 μl), and incubated at 60° for 60 min. Four microliters of loading buffer (30%, w/v, glycerol, 20 mM EDTA, 0.08% bromphenol blue) were added, and the complete sample was electrophoresed on 1% agarose slab gels containing 0.5 μg/ml ethidium bromide, with 40 mM Tris acetate, 1 mM EDTA, pH 8.5, as the runnning buffer.

Fluorescence and electron microscopy. For the morphological studies, the cells were washed twice in PBS, fixed in cold ethanol, and stained with propidium iodide (10 μg/ml, in PBS). For electron microscopy, thymocytes were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, postfixed with 1% osmium tetroxide, and embedded in Epon. The thin sections were mounted on nickel grids and examined by electron microscopy (Siemens, Alpharetta, GA) after staining with uranyl acetate and lead citrate.

Thymocyte viability by MTT assay. Cell viability after incubation for different times was assessed by a tetrazolium-based colorimetric assay. Thymocytes were incubated with 50 μl of a 5 mg/ml MTT solution (Sigma) in PBS for the last 4 hr of culture in 96-well plates (Falcon). After supernatant removal, cellular pellets were dissolved in 200 μl of 0.4 N HCl/isopropanol solution by agitation, and absorbance at 570 nm was measured using a Multiscan reader.

***In vivo* Dox or Dox plus IL-2 treatment of naive or tumor-bearing animals.** Mice were inoculated with 5×10^4 EL4 lymphoma cells on day 0. Dox was injected intravenously on day 5 in two different doses, 5 and 10 mg/kg. Dox-induced effects were assessed in tumor-bearing animals on day 8. In the combination treatment studies, Dox (5 mg/kg, intravenously) was injected on days 1 and 8 and IL-2 (2 μg, intraperitoneally, twice each day) was injected on days 9–40.

Histology. Animals were sacrificed at different times after Dox injection (4 hr to 5 days), and their thymi were removed and fixed in 4% buffered formalin. Paraffin sections were stained with hematoxylin and eosin and examined microscopically.

Phenotype analysis. One million thymocytes were washed, suspended in 50 μl of PBA (PBS containing 0.5% bovine serum albumin and 0.1% sodium azide), and placed on ice. Ten microliters of whole mouse immunoglobulin (Cappel, Cochranville, PA) were added to competitively block the Fc receptor. Ten microliters (1 μg/10^6 cells) of FITC-labeled anti-CD3 (clone 145–2C11; Boehringer Mannheim, Indianapolis, IN), TC-conjugated anti-CD4 (clone YTS 191.1; Caltag, San Francisco, CA), and phycoerythrin-conjugated anti-CD8a (clone YTS 169.4; Caltag) were added, and cells were incubated for an additional 20 min on ice. After two washings in PBA, cells were fixed in 2% formaldehyde (ultrapure; Polysciences, Warrington, PA). Anti-CD3 was substituted by FITC-anti-CD44 (clone 1M7; Pharmingen, San Diego, CA), FITC-anti-TCR-αβ (Caltag), or FITC-anti-TCR-γδ (Caltag) in parallel samples. The corresponding fluorochromes conjugated to rat or hamster immunoglobulins (Caltag) were used as isotype controls. To control for possible fluorescence due to Dox uptake *in vitro* and *in vivo*, control thymocytes with equivalent drug amounts added were either stained immediately or kept at 4° overnight to eliminate Dox efflux before analysis. A FACScan (Becton-Dickinson) was used for three-color immunofluorescence analysis. Ten thousand events were acquired in list mode. Lysys II and Paint-A-Gate (Becton Dickinson) programs were used for subset enumeration.

Results

Morphology of Dox-induced effects on thymocytes *in vitro*. Fluorescence microscopic comparison of cells that had been incubated in medium alone, in medium plus Dex, or in medium plus one of three different concentrations of Dox revealed that each sample contained a different percentage of the total cell population that was apoptotic (Table 1). The majority of cells (69%) in samples incubated with 0.1 μM Dex were undergoing apoptotic changes by 24 hr. There was detectable apoptosis in cells incubated in medium alone (12.3%) or with 0.05 μM Dox (9.3%). Incubation with 0.5 μM Dox increased the percentage (38%) of apoptotic nuclei. Cells exposed to the highest (5 μM) concentration of Dox showed fewer apoptotic nuclei (3%) than did the control cells. Nevertheless, the number of cells remaining after a 24-hr exposure to 5.0 μM Dox was considerably smaller than that remaining after exposure to the two lower drug concentrations, indicating that some form of

TABLE 1

Morphological determination of apoptotic nuclei in thymocytes after exposure to Dex or Dox

After 24-hr exposure to the indicated agents, thymocytes were stained with propidium iodide (10 μg/ml) and nuclear morphology was evaluated by fluorescence microscopy. Homogeneous bright nuclei were considered apoptotic. Two hundred cells were counted for each sample.

Incubation medium	Apoptotic nuclei
	%
Control	12.3
Dox, 0.05 μM	9.3
Dox, 0.5 μM	37.8
Dox, 5.0 μM	3.0
Dex, 0.1 μM	68.8

Dox-induced killing had occurred. There were no detectable increases in the size of the cells or nuclei in samples incubated with Dox at any of the three concentrations. This is consistent with the fact that such increases have been described only in rapidly cycling cell lines (8). Electron microscopic examination (Fig. 1) confirmed that Dox-treated thymocytes developed the characteristic apoptotic morphology, i.e., unipolar chromatin condensation to the nuclear membrane in dense patches and crescents, zeiosis, and electron-dense cytoplasm.

Estimation of chromatin degradation induced by Dox. The distinct subdiploid region present in DNA distribution histograms of murine thymocytes represents the fraction undergoing apoptotic DNA degradation (1, 2, 16, 17). Thymocytes incubated for 24 hr in medium alone (Fig. 2A) or with a low concentration (0.05 μM) of Dox (Fig. 2B) formed two peaks, i.e., a high diploid peak (G_0/G_1) and a low subdiploid peak (A_0) representing degraded chromatin. Treatment of thymocytes with 0.5–1.0 μM Dox (see, for example, 0.75 μM Dox in Fig. 2C) or 0.1 μM Dex (Fig. 2E) increased the subdiploid (apoptotic) region (A_0 = 46% and A_0 = 67%, respectively), compared with incubation in medium alone (A_0 = 15%) (Fig. 2A) or with 0.05 μM Dox (A_0 = 14%) (Fig. 2B). At high Dox concentrations a subdiploid peak was not seen (Fig. 2D). Although results entirely similar to those described above were obtained after 18-hr exposure to the agents (data not shown), to determine whether the high Dox concentration induced detectable apoptosis at an earlier time point the following experiment was carried out (Table 2). Thymocytes were plated, exposed to Dox (5 μM), Dex (0.1 μM), or medium alone, and incubated for 2, 4, 6, 8, 12, or 18 hr, and the percentage of apoptotic cells was established by FCM. Dox (5 μM) did not induce apoptosis markedly above spontaneous levels (i.e., in medium alone) at any time point examined and, in fact, the level of apoptosis seen was below spontaneous levels at several time points. Dex, added as a positive control, caused increasing apoptosis from 2 hr to 18 hr.

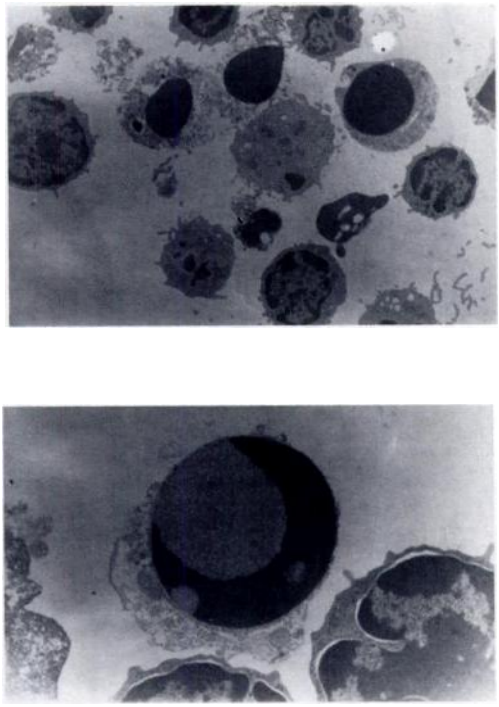

Fig. 1. Electron microscopy of Dox-treated cells. Morphological view of the thymocytes using the electron microscope (*upper*, 6000×; *lower*, 10,000×), demonstrating the presence of characteristic features of apoptosis after exposure to 1.0 μM Dox for 24 hr.

As demonstrated in Fig. 3A, Dox-induced degradation of thymocyte DNA was concentration dependent over the concentration range of 0.01–2.5 μM. There was a direct correlation between the percentage of the population in the subdiploid region (Fig. 3A) and the number of surviving cells (evaluated by the MTT colorimetric assay) over this concentration range (Fig. 3B). Chromatin degradation was abruptly reduced to almost undetectable levels at Dox concentrations of 2.5 μM and above (Fig. 2D). The surviving cell fraction determined by MTT assay after exposure to Dox in this concentration range was small, although still detectable (e.g., ranging from 3 to 12% at 5 μM Dox) (Fig. 3B). These data suggest that Dox exposure initates apoptosis over a narrow drug concentration range (0.05–2.5 μM). At concentrations above 2.5 μM, Dox-induced cell death occurs without detectable DNA degradation, indicating that a Dox-mediated cytotoxic mechanism other than apoptosis is operative.

Agarose gel electrophoresis of DNA fragments. The DNA fragmentation associated with apoptosis is characterized by cleavage of the DNA at regular intervals, as opposed to the random fragmentation associated with necrosis. Gel electrophoresis of DNA isolated from apoptotic cells produces a char-

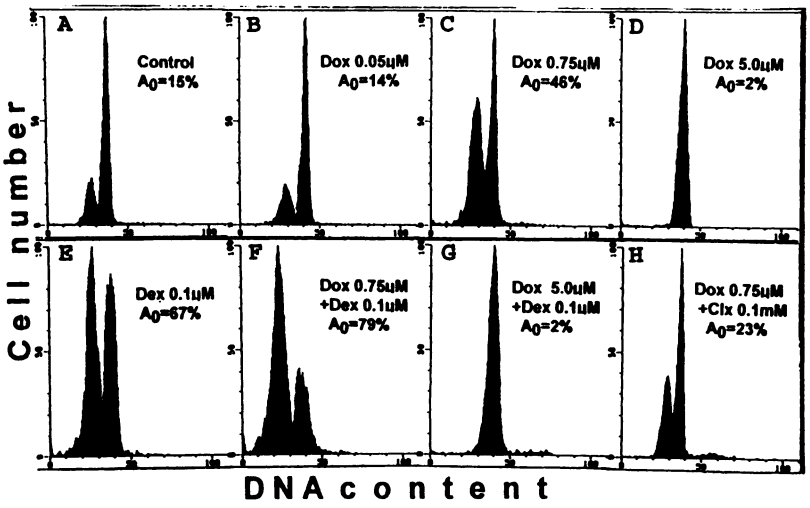

Fig. 2. FCM analysis of chromatin degradation in cells after 24-hr exposure to the various agents. The agents, at the indicated final concentrations, were added at the initiation of cultures. The percentages of cells (A_0 values) in peaks with diminished amounts of DNA are shown. Ten thousand events were acquired for each analysis.

TABLE 2

Thymocyte apoptosis after incubation with Dox or Dex for different periods of time

Samples of thymocytes incubated for different periods of time with the indicated agents were stained with propidium iodide and examined for apoptosis by FCM analysis.

Incubation time	Apoptotic cells		
	No treatment	Dex (0.1 μM)	Dox (5.0 μM)
hr		%	
0	2.3		
2	8.3	11.7	9.9
4	6.5	19.4	6.8
6	7.8	35.8	11.1
8	6.1	37.8	11.4
12	11.5	44.8	7.4
18	18.0	60.9	8.8

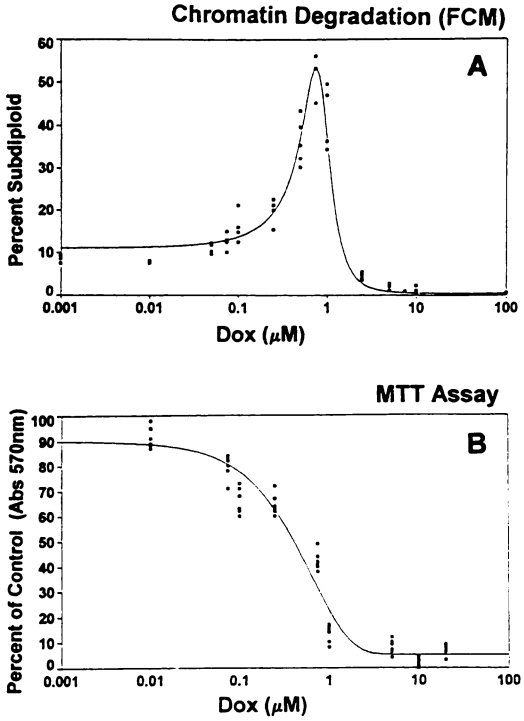

Fig. 3. Quantitation of chromatin degradation and total number of viable cells in Dox-treated thymocyte cultures. Thymocytes cultured with different concentrations of Dox were examined for percentage of subdiploid nuclei (chromatin degradation) (A) and for viability in the MTT assay (B). The percentage of viable cells was defined as the ratio of the absorbance (λ = 570 nm) of experimental samples to that of control samples.

acteristic DNA ladder consisting of fragments that are multiples of 200 base pairs. Gel electrophoresis of DNA isolated from Dox-treated thymocytes demonstrated laddering patterns (Fig. 4) for those samples predicted to contain apoptotic cells, based on the presence of the subdiploid FCM peak seen in parallel samples (Fig. 2). Thus, a clear ladder of degraded DNA was found in control thymocytes and in specimens incubated with 0.5 μM but not 5 μM Dox. Visual inspection of the gels revealed that treatment of the thymocytes with either Dox or Dex increased PCD above control levels; however, the extent of PCD could not be quantitated by this method.

Protection of thymocytes from Dox-mediated apoptosis by inhibition of protein or RNA synthesis. PCD is in most cases a process that requires active protein and RNA synthesis (1, 18). Glucocorticoid-induced thymocyte apoptosis

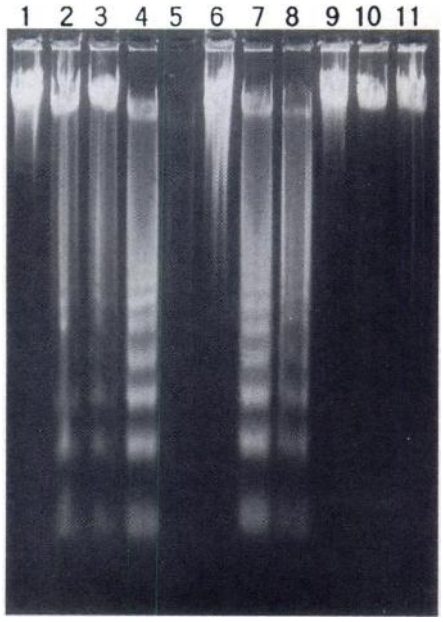

Fig. 4. Agarose gel electrophoresis of cellular DNA. Cells were incubated, except as noted, at 37° for 18 hr with various agents, as follows: *lane 1*, medium only (control) at 4°; *lane 2*, control at 37°; *lane 3*, 0.05 μM Dox; *lane 4*, 0.5 μM Dox; *lane 5*, no sample loaded; *lane 6*, 5.0 μM Dox; *lane 7*, 0.1 μM Dex; *lane 8*, 0.5 μM Dox and 0.1 μM Dex; *lane 9*, 5.0 μM Dox and 0.1 μM Dex; *lane 10*, 0.5 μM Dox and 50 μg/ml Clx; *lane 11*, 0.5 μM Dox and 2 μg/ml Act.

is prevented by inhibition of protein synthesis with Clx (2, 16, 19) or mRNA synthesis with Act (1, 19). In the present study, thymocytes continuously incubated for 24 hr with Dox plus either agent (Clx or Act) had markedly lower subdiploid populations than did cells incubated with Dox alone (results with Dox plus Clx are shown in Fig. 2H). Thymocytes incubated with Clx or Act alone exhibited 8.4% and 7.0% apoptotic cells, respectively, versus 12% apoptotic cells in medium alone. Similarly, Clx and Act reduced the detectable Dox-induced DNA laddering (Fig. 4, *lanes 10* and *11*).

Prevention of Dex-induced apoptosis by exposure to high doses of Dox. When thymocyte samples were continuously incubated with Dex (0.1 μM) plus Dox (0.001–1.0 μM), additive effects on chromatin degradation were seen (Fig. 2F). In contrast, high doses of Dox (2.5–10 μM) prevented Dex-associated PCD (Fig. 2G). Although in the presence of 2.5–10 μM Dox the cell counts were significantly reduced at 24 hr, 5–15% of the starting population was found to be intact at that time and 66% of those cells excluded trypan blue. Thus, these results are consistent with those obtained in the MTT assay and indicate that 3–10% of the starting population remained viable at 24 hr. It is of interest that apparently none of those cells were triggered to undergo apoptosis when exposed to Dex plus Dox (Figs. 2G, 3A, and 4, *lane 9*). This finding is consistent with the possibility either that the entire preapoptotic population is eliminated by Dox-induced necrosis or that Dox, in this concentration range, renders preapoptotic cells nonresponsive to Dex-mediated PCD initiation signals.

Evidence that the $CD3^{-/lo}CD4^{+}CD8^{+}$ thymocyte population is the most susceptible to Dox-induced apoptosis. FCM analysis of the triple-labeled (FITC-anti-CD3, phycoerythrin-anti-CD8, and TC-anti-CD4) cells revealed that $CD3^{-/lo}CD4^{+}CD8^{+}$ thymocytes were depleted to a greater extent

than any other subset after exposure to 0.5 μM Dox or 0.1 μM Dex (Table 3). Although the other three subsets increased slightly as percentages of the total lymphocyte population, in absolute numbers of cells (data not shown) the double-negative ($CD3^-CD4^-CD8^-$) and single-positive ($CD3^{hi}CD4^+CD8^-$ or $CD3^{hi}CD4^-CD8^+$) subsets were also reduced, indicating that these subpopulations undergo apoptosis as well. Exposure to a high concentration (5 μM) of Dox resulted in percentages of the DP subset equal to those seen before incubation and higher than those seen in populations incubated either in medium or with the lower concentrations of Dox. This finding is consistent with the total lack of detectable apoptosis. Thus, thymocytes undergoing PCD [either spontaneously or induced by a low Dox concentration (0.5 μM)] preferentially lost the DP population, whereas necrosis affected all populations equally.

Dox-induced redistribution of thymocyte populations *in vivo*. The DP cortical thymocytes have been reported to undergo apoptosis *in vivo* during TCR/CD3 pathway activation after antigenic stimulation (20), anti-CD3 treatment (21), or accelerated allograft rejection (22). Examination of histological sections of thymus from untreated control mice and from mice treated with 5 or 10 mg/kg Dox (single intravenous injection) 5 days earlier showed dose-dependent Dox-induced depletion of the cortical areas (Fig. 5, A versus B or C). In fact, at the 10 mg/kg dose, the medullar region appeared to be even more cellular than the cortical region (Fig. 5C). Epithelial cells forming Hassall's corpuscles in the medulla were unaffected. FCM analysis revealed that Dox injection (5 or 10 mg/kg, intravenously, on day 5) (Fig. 6) reduced DP populations more than *in vitro* Dox exposure (0.5 μM for 24 hr) (Table 3). This may be due to rapid elimination of apoptotic cells *in vivo* by phagocytes. In support of this possibility, thymocytes obtained 48 hr after Dox injection and then cultured for an additional 48 hr showed significantly higher levels of apoptosis than did cultured thymocytes from untreated mice (data not shown). Among the other subpopulations analyzed, $CD3^+$(TCR-γδ)$CD4^-CD8^-$ cells showed relative resistance (Table 4) to Dox. The $CD44^+CD4^-CD8^-$ population was increased 1.3-fold, in absolute numbers. The latter finding is most likely due to facilitated immigration of early immature T cell precursors from the bone marrow, because the CD44 molecule is expressed by T cell progenitors and, through its role as an adhesion molecule, is thought to facilitate their entry into the thymus from the blood vessels (23). Finally, when the DP thymocyte population was examined during the 3-week period after a single Dox injection (5 mg/kg), it showed an increase back to normal levels and even a rebound above control values (data not shown).

TABLE 3

Thymocyte subset profiles after incubation with drugs for 24 hr

Samples of thymocytes, either before or after incubation for 24 hr with the various agents, were stained with FITC-conjugated anti-CD3, phycoerythrin-conjugated anti-CD8, and TC-conjugated anti-CD4. Coexpression of CD3 on single-positive cells was used to assess mature populations. DP cells were negative for CD3 or expressed low levels of the molecule.

Incubation medium	Subsets			
	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^-CD8^-$
	%			
Before incubation	83.1	9.8	4.1	3.0
Medium alone	78.2	11.7	5.0	5.1
Dox, 0.05 μM	77.8	11.9	5.1	5.2
Dox, 0.5 μM	63.7	16.1	10.5	9.7
Dox, 5.0 μM	83.3	8.7	4.3	3.7
Dex, 0.1 μM	50.4	25.5	13.0	11.1

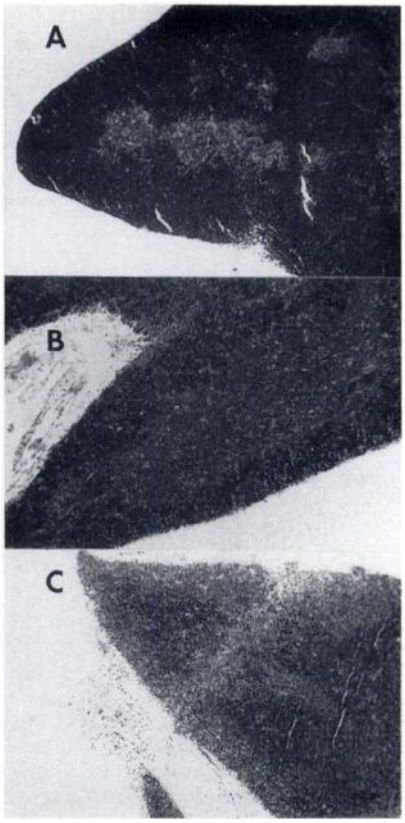

Fig. 5. Histological sections of thymi from C57BL/6CrN mice. A, Untreated control; B, 5 mg/kg Dox-treated mouse; C, 10 mg/kg Dox-treated mouse. Thymi were removed 5 days after intravenous Dox administration. Paraffin sections were stained with eosin and hematoxylin (100×).

Lack of prevention of DP thymocyte depletion *in vivo* by the combination of Dox and IL-2. Different cytokines (IL-2, IL-3, and IL-4) have been reported to protect against apoptosis induced by cytotoxic drugs or radiation (1, 24, 25). The capacity of the T cell growth factor IL-2 to protect thymocytes from Dox-induced apoptosis *in vivo* was examined. Using a previously reported effective and safe regimen (26), IL-2 was injected twice daily starting 24 hr after Dox (5 mg/kg, intravenously) treatment of animals bearing EL4 lymphoma. Neither the tumor itself nor the administration of IL-2 affected any thymocyte subset; however, Dox at this dose reduced the percentage of DP thymocytes to a minor extent (Table 5). Unexpectedly, further depletion of the DP population in the thymus was seen in animals receiving Dox and IL-2 in combination. Nevertheless, 55% of the animals receiving this combination were long term survivors (>60 days) and 100% of the long term survivors were able to survive reimplantation of EL4 tumor cells on day 60 (Table 5). These findings indicate that the treatment induced marked depletion of the cortical DP thymocytes, seen on day 12, but did not have a negative impact on the induction of a significant survival fraction or the development of antitumor memory. Additional studies will be required to establish whether there is a cause-effect relationship between these events.

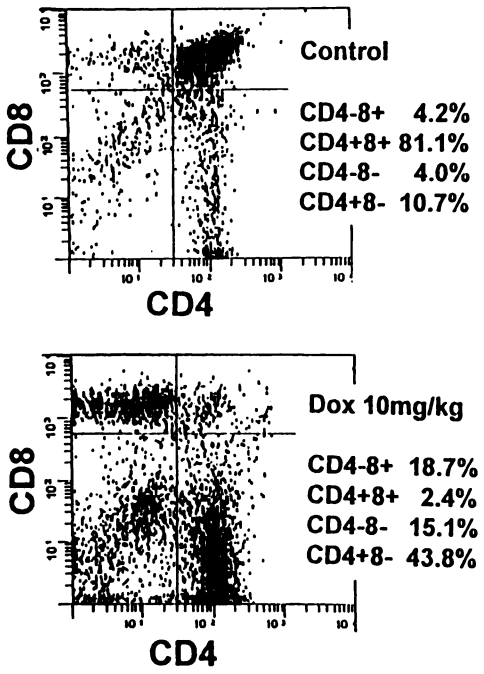

Fig. 6. Dual-color FCM analysis of CD4 and CD8 expression in thymocytes. Thymocytes were obtained from untreated (control) and Dox-treated (5 days after 10 mg/kg Dox injection, intravenously) C57BL/6CrN mice. Representative specimens from one of four separate experiments (five animals/group) are shown. Ten thousand events were acquired to calculate the subset percentages.

TABLE 4

Immunophenotypic profile of thymocytes 5 days after Dox injection

Mice were sacrificed on day 5 after Dox injection (5 or 10 mg/kg, intravenously, single push). Thymocytes were stained with FITC-conjugated anti-TCR-$\alpha\beta$, phycoerythrin-conjugated anti-CD8, and TC-conjugated anti-CD4. Alternatively, FITC-conjugated anti-TCR-$\gamma\delta$ or CD44 was used for triple-stained samples. Absolute numbers of cells in each subset were derived from total cellularity (determined from hemocytometer counts), and the percentage for each subpopulation was determined by three-color FCM. A representative experiment of three is shown.

Subsets	Cell number (10^6/thymus)		
	Control	Dox	
		5 mg/kg	10 mg/kg
$CD3^-CD4^-CD8^-$	8.23	6.25 (76%)[a]	6.04 (73%)
$CD3^+CD4^-CD8^-$ (TCR-$\gamma\delta$)	1.42	1.39 (98%)	1.40 (99%)
$CD3^-CD4^+CD8^+$	69.26	27.85 (40%)	0.44 (0.6%)
$CD3^{lo}CD4^+CD8^+$ (TCR-$\alpha\beta$)	93.88	38.58 (41%)	0.52 (0.6%)
$CD3^+CD4^+CD8^-$	19.16	17.06 (89%)	17.52 (91%)
$CD3^+CD4^-CD8^+$	9.41	7.90 (84%)	7.50 (80%)
$CD44^+CD4^-CD8^-$	1.89	ND[b]	2.53 (134%)

[a] Values in parentheses, percentage of control.
[b] ND, not determined.

Discussion

As shown in this report, cells undergoing "spontaneous" apoptosis were detectable in murine thymocyte populations after 24 hr in culture. Increased levels of apoptosis were found after 24-hr exposure to Dox but only over a narrow concentration range (0.1–1.5 μM). Higher concentrations of Dox (2.5–10 μM) caused extensive thymocyte lysis, most likely by necrosis, with no evidence of apoptosis even at the level of spontaneous apoptosis seen in the controls. In this higher concentration range, Dox also completely blocked Dex-initiated PCD. A variety of clinically used agents, such as steroids (1, 16, 17), radiation (17, 24), antagonists of steroid hormones (27), topoisomerase inhibitors (2, 6, 11), and certain other cytotoxic agents (15, 24), have recently been demonstrated to kill cells by apoptosis. In mice, the uptake of Dox (5 mg/kg, intravenously) into the thymus peaks on days 2–3, at a level quite close to that necessary to initiate thymocyte apoptosis *in vitro* (28). In the clinical situation, based on the known pharmacokinetics of Dox, prolonged exposure to Dox occurs primarily during the second phase of drug elimination, as Dox is slowly released from tissue stores. During this phase, sustained plasma levels are close to 0.1 μM (29), a concentration that induces murine thymocyte apoptosis. However, the threshold for initiating apoptosis in the human system may differ.

The chromatin degradations induced by Dox and Dex apparently use similar biochemical pathways, because Act, a transcription inhibitor, and Clx, a protein synthesis inhibitor, prevent Dox-associated apoptosis in a manner similar to that reported for Dex (1, 16, 19). It has been shown in some experimental systems, however, that Clx and Act cannot protect cells from PCD and, in fact, these agents may even initiate apoptosis, depending upon the concentration (30, 31). Thus, the mechanism of irreversible DNA fragmentation may differ from one experimental model to another. Similarly, Dox has been shown to induce PCD in some cell types (3, 4) but not in others (5, 6). The findings reported here with thymocytes are in agreement with the data reported by Ling *et al.* (32), demonstrating that 1.0 μM Dox effectively induced apoptosis, whereas neither higher nor very low concentrations of Dox produced DNA cleavage in the P388 cell line. It is of interest that the capacity to induce PCD in certain cells varies with the anthracycline tested (5, 32), but it is not clear at present whether these differences are attributable to variables not yet examined (e.g., differences in concentration range, characteristics of each individual anthracycline, or structure/activity characteristics).

Hotz *et al.* (33) demonstrated recently that disappearance of the subdiploid population after exposure of HL-60 cells to high (1 mM) concentration of fostriecine was due to immediate killing (i.e., necrosis). This would also seem to be the case after exposure to 2.5–10 μM Dox in the present study, because no cells undergoing apoptosis were observed after either 18 or 24

TABLE 5

Percentage of DP thymocytes in EL4 lymphoma-bearing animals undergoing treatment with Dox, IL-2, or a combination of both agents

Mice bearing EL4 ascitic leukemia were treated with Dox (5 mg/kg, intravenously on days 1 and 8), IL-2, (2 μg, intraperitoneally, twice each day, days 9–40), or a combination of both regimens. On day 12 animals (10/group) were sacrificed and thymi were analyzed for expression of accessory molecules as described for Table 3.

Therapy	$CD4^+CD8^+$	MST[a]	LTS[b]	Tumor rechallenge immunity[c]
	%	*days*	%	%
None	51.2	12	0	
Dox	37.1	23	0	
IL-2	49.1	26	0	
Dox + IL-2	24.3	[d]	55	100

[a] MST, median survival time.
[b] LTS, long term survivors (those animals that survived >60 days were considered long term survivors).
[c] After day 60, long term survivors were rechallenged with 5×10^4 EL4 cells (at least 500 times the lethal dose).
[d] Median survival time could not be determined because more than half of the animals survived for 60 days or more.

hr, although viable cells as well as dead cells were still present at both time points. Interestingly, under these conditions the added presence of Dex also failed to initiate apoptosis in the surviving fraction. This could be explained if both drugs were targeting the same subset(s) of the thymocyte populations. Recently, the existence of a preapoptotic population in rat thymocytes was reported (34); this might be a target for both drugs. It is possible that those "preapoptotic changes" render cells more fragile, such that they are more susceptible to signals inducing either apoptotic or necrotic cell death. This possibility, however, does not seem consistent with the observations (Table 3) that the subset profile of the intact cells 24 hr after the addition of 5 μM Dox was identical to that of the starting population and that 83% of the cells were DP.

Apoptosis is a physiological process for selective cell deletion. Apoptotic cells often exist directly adjacent to unaffected neighboring cells. The selectivity in the thymus is known to be immunophenotype related; the cortical DP thymocyte subset is the most susceptible (1, 20–22). These DP thymocytes have been shown to be devoid of Bcl-2 expression, whereas both the less mature double-negative and the more mature single-positive thymocytes do express Bcl-2 (35). Because Bcl-2 has recently been shown to function in an antioxidant pathway to prevent apoptosis (36) and the capacity of Dox to produce reactive oxygen species is well known (37), it can be suggested that Dox may induce apoptosis by this pathway. As reported here, the effect of Dox on DP thymocytes was pronounced, both *in vivo* and *in vitro*, but few apoptotic cells were found *in vivo*. Presumably this was due to phagocytic clearance of cells undergoing apoptosis during the 5-day period after drug administration and before the assay. Nevertheless, in both cases the single-positive and double-negative subsets showed considerably more resistance to Dox than did the DP subpopulation. This profile is somewhat similar to that seen after cyclophosphamide (38) or steroid (39) administration. The behavior of the thymic $CD3^+(TCR\text{-}\gamma\delta)CD4^-CD8^-$ cells was similar to that reportedly found after cyclosporin A administration (40), and the increase in the absolute numbers of $CD44^+CD4^-CD8^-$ cells is consistent with the rapid recruitment of T cell progenitors from the bone marrow. The inability of IL-2 to protect cortical thymocytes from entering PCD *in vivo* might seem to be in contradiction to the capacity of IL-2 to induce thymocyte proliferation. Migliorati *et al.* (41) demonstrated, however, that *in vitro* exposure of mouse thymocytes to IL-2 (400 units/ml) can facilitate apoptosis. It is extremely unlikely, however, that this concentration of IL-2 was attained in the thymus in the present study, especially because of the short half-life of IL-2 and the poor thymic vascularization.

Thymic atrophy during chemotherapy and regrowth during the recovery phase is a relatively common phenomenon that occurs in about 90% of patients (42). The fact that thymic regrowth may be a positive prognostic sign has been suggested (43). The present study shows that the principal thymic cells undergoing apoptosis in mice treated with Dox or Dox plus IL-2 are the DP cortical thymocytes. There is also an apparent association between animal survival rates, rechallenge immunity, and DP depletion. However, this should not be considered inconsistent with the possibility of T cell involvement (production of tumor-reactive clones) in tumor recognition and eradication. In fact, extensive DP apoptosis has been reported in animals undergoing accelerated heart allograft rejection (22) and after certain types of antigenic stimulation (20). Even under physiological conditions, elimination of a large proportion of nonselected cells (negative selection for deletion) occurs; this is followed by the influx of new precursors. Such deletion, therefore, would be consistent with the hypothesis that this is a mechanism to "make room" for new waves of positively selected progenitors (44). At present it is not possible to definitely explain how Dox plus IL-2 therapy induces immunological memory against syngeneic tumors (26). It is tempting to speculate that Dox-induced depletion of DP cells within the thymus results in the maturation of increased numbers of new clones of positively selected progenitors, which are then sustained by IL-2 in the periphery, and, therefore, that increased numbers of cells are available for activation against tumor-derived peptides in the context of self major histocompatibility complex.

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